Polyethylene glycol-based precipitation method for the isolation of urinary exosomes from buffaloes and characterization by Transmission Electron Microscopy

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Abstract

Urinary exosomes are nano-sized extracellular vesicles which are released into the urine during the physiological and pathological conditions. Exosomes contain protein, mRNA and microRNA representative of their cell type of origin and their concentration may vary according to the biochemical state of the individual animal. Isolation and characterization of exosomes are necessary before urinary exosome biomarkers can be found. For the first time, a polyethylene glycol (PEG)- based precipitation method was standardized for the isolation of exosomes from the urine of buffaloes at the estrus phase. The isolated urinary exosomes were characterized by transmission electron microscopy which revealed the vesicles are of less than 100nm size and had the property to co-precipitate. Upon isolation of total RNA and protein from these urinary exosomes by trizol method, sufficient RNA and protein yield was obtained. More intense protein bands were obtained on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) from urinary exosomes isolated by the PEG precipitation when compared to the ultracentrifugation method. As a result, our PEG precipitation method provides a rapid, scalable, and efficient substitute for the ultracentrifugation method in the process of isolating exosomes from buffalo urine. The use of urinary exosomes isolated by this PEG precipitation method for proteomic and transcriptomic studies needs further validation such that the present study can aid in biomarker studies.

Key Words: Urinary exosomes, Polyethylene glycol, Transmission electron microscopy, Buffalo, Biomarkers

Introduction

Exosomes are approximately 30 to 150 nm sized extracellular vesicles (EVs) that are secreted by cells of renal and urogenital tract epithelium. According to the International Society for Extracellular Vesicles (ISEV) established in the year 2011, "EVs are non-replicative spherical particles naturally released from the cell into the extracellular space and are delimited by a lipid bilayer". During both normal and pathological situations, several cell types release their cargo into the extracellular space, which is indicative of the EVs' cellular origin (Cossetti *et al*., 2014). Numerous biological fluids, including blood, milk, saliva, amniotic fluid, urine, cerebrospinal fluid (CSF), semen, breast milk, and malignant ascites, have been found to contain EVs (Lasser *et al*., 2011).

Based on the current state of knowledge of their biogenesis, EVs are broadly classified into three subtypes as exosomes, ectosomes and apoptotic bodies (ABs) (Kalra *et al*., 2016). Among the EV subtypes, exosomes have been and are extensively studied. Intraluminal vesicles (ILVs) called exosomes start in the endosomal compartment. ILVs are formed by inward budding of the early endosomal membrane, sequestering proteins, lipids, and cytosol, to degrade, recycle or exocytose their content. Early endosomes then mature into late endosomes and the accumulation of multitude of ILVs leads to the formation of multi vesicular bodies (MVBs), which fuse with the plasma membrane to release the exosomes into the extracellular space (Juan and Furthauer, 2015).

Urine is a non-invasive source of biological fluid and reflects the physiological status of the mammals (Bathla *et al*., 2015). Though the protein concentration in normal urine is usually very low, the interest in using urine for diagnostic applications is increasing rapidly due to its simple and non-invasive collection compared to other biological fluids. Urinary exosomes carry cell-related proteins, DNA, mRNA, miRNA, lipids involved in cell communication, cell migration, angiogenesis, and immune regulation (Erdbrugger and Le, 2015). Very few studies have been reported on the buffalo urinary proteome/urinary exosome proteome and small RNA as a potential source for biomarker discovery in physiological phases like estrus period and pregnancy (Srinivasan *et al*., 2020; Hebbar *et al*., 2021).

There are several methods to isolate urinary exosomes (He *et al*., 2019). Though, Ultracentrifugation (UC) remains the gold standard for EV isolation from various biological fluids, the technique itself requires skilled personnel and is time-consuming. Therefore, the aim of the present study was to standardize a polyethylene glycol (PEG) based precipitation method for the isolation of urinary exosomes from buffaloes and their characterization by transmission electron microscopy (TEM).

Materials and Methods

Collection of urine samples

Mid-stream urine samples were collected from healthy cycling Murrah buffaloes at the estrus phase. The urine samples were strained through a muslin cloth and transported to the laboratory in cold chain. The samples were then stored at -80° C until further analysis by adding phenyl methyl sulfonyl fluoride (PMSF) as a preservative at 0.01%.

Isolation of Urinary Exosomes by Polyethylene glycol Precipitation

Several PEG-based exosome isolation techniques that were published were referred, (Shin *et al*., 2015; Konoshenko *et al*., 2018; Lv *et al*., 2018) and the proportion of PEG that should be utilized to precipitate exosomes from buffalo urine was standardized as explained below.

Thawed urine samples (30ml) were centrifuged at 3000g for 10min at 4°C. The supernatant was collected into a new 50ml falcon and 12.5ml of 24% PEG (PEG 6000 from Hi Media) solution made in 375 mM NaCl was added. The mixture was vigorously mixed and incubated in a cold chamber at 4°C for the entire night. The solution was then transferred to Oakridge tubes, centrifuged at 4000g for one hour at 4°C (KUBOTA, High Speed Refrigerated Centrifuge 6500). The resultant pellet was resuspended in 100µl of DEPC Rx PBS and stored at -80°C until further use.

Characterization of urinary exosomes by TEM

The urinary exosomes isolated by the above-described PEG precipitation method and resuspended in DEPC Rx PBS were thawed on ice and a drop of exosome suspension was added to carbon coated grids and allowed for attachment for 1min. Then negative staining was done to visualize the exosomes, as per the procedure described by Rikkert *et al*. (2019) with slight modifications, by adding carefully three drops of 1% uranyl acetate. The first drop (1% uranyl acetate) was allowed for 10 sec and then blotted on air using Whatman filter paper No.1. The second drop was allowed for 10 sec and then blotted on air using Whatman filter paper No.1. The third drop was allowed for 30 sec and then blotted by just touching the periphery of the grid using Whatman filter paper No.1. After that, the coated grid was left to dry for five to ten minutes. The grid was then placed onto the holder carefully by using a forceps. The holder was then inserted into TEM (Talos L120C, ThermoScientific, Electron Microscopy Facility, Division of Biological sciences, Indian Institute of Science, Bangalore) and imaged using 200nm filter. The exosomes' dimensions and morphology were noted.

Isolation of total RNA from urinary exosomes by using Trizol

Total RNA from urinary exosomes isolated by the above method and characterized by TEM was carried by Trizol method as per Manasa *et al*. (2020) with minor modifications. The exosome suspensions were thawed on ice and 1ml Trizol reagent was added to the samples. Using a 1ml Tuberculin syringe the exosome suspension was dissolved and gently disrupted in Trizol to release the RNA and contents out of the membrane vesicles. After 10 minutes of incubation at room temperature (RT), the mixture was spun for five minutes at 12,000 rpm. 200µl of chloroform was added to the supernatant and stirred for 15 seconds. The bottommost pinkish protein layer, a whitish interphase holding the DNA, and the upper aqueous phase carrying the RNA were the three phases that emerged from centrifuging the suspension at 14000 rpm for 15 minutes at 4°C after it had been incubated at room temperature for two to three minutes. The upper aqueous phase was taken in a fresh Eppendorf tube and 500 µl of 100% isopropanol was added, incubated at RT for 10 min. The mixture was spun at 12000 rpm for 10 min at 4°C. To the pellet 1 ml of 75% ethanol was added and spun at 7500 rpm for 5min at 4°C. The alcohol was discarded and air dried for 10-30 min. The RNA pellet was then resuspended in 20-50 µl of DEPC Rx water.

Estimating the concentration and purity of RNA from urinary exosomes

The concentration and purity of total RNA isolated from urinary exosomes by the PEG based method was estimated using nanodrop by measuring the absorbance at 260nm and absorbance ratio of 260/280nm.

Isolation of protein from urinary exosomes by using Trizol, estimating the concentration of protein and SDS-PAGE for resolving the urinary exosome proteins

The bottom pinkish layer obtained in the phase separation during RNA isolation was taken in an eppendorf tube and isopropanol (1.5 times) was added to it, mixed and incubated at RT for 10 min. The suspension was then centrifuged at 12,000g for 10 min at 4°C. After discarding the supernatant, the pellet was reconstituted in 75% ethanol and allowed to sit at room temperature for 20 minutes. The suspension was centrifuged at 7500g for 5 minutes at 4°C. After three iterations of the ethanol washing described above, the pellet was resuspended in 100% ethanol for 20 minutes at room temperature. For five minutes, the mixture was centrifuged at 7000g at 4°C.

The protein pellet obtained was dried at RT for 5 min and resuspended in 5µl of PBS and equal volume of Laemmli lysis buffer (Van Deun *et al*., 2014) was added. According to Van Deun *et al*. (2014), the urine exosomal proteins (60µg) were resolved on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue after the protein concentration was estimated using the Bradford assay (Bio-Rad).

Results

The morphology and the size of urinary exosomes isolated from buffaloes at estrus phase by PEG based method and characterized by TEM is shown in figure 1 (1A, 1B and 1C). In all the Figs (1A to 1C), there is a bar indicating size in nm to aid in estimating the diameter of the vesicles focused in the particular field/figure. All the figures show negatively stained urinary vesicles ranging from 50-100nm in diameter.

The average concentration of total RNA (n=3) isolated from urinary exosomes at estrus phase by the PEG based method was 336ng/µl and the purity (Absorbance ratio at 260/280nm) was 1.78. The average concentration of protein from urinary exosomes (n=3) was found to be 0.9mg/ml. The protein bands of urinary exosomes resolved on SDS-PAGE and stained with Coomassie brilliant blue (CBB) is shown in figure 2. The proteins predominantly identified on SDS-PAGE by CBB staining in urinary exosomes isolated by PEG based method were having molecular weights between 180 to 115 KDa. Distinct protein bands were also identified at around 100KDa and 50 KDa. While distinct protein bands were not detected at around 50 KDa and between 180 to 115 KDa in urinary exosomes isolated by ultracentrifugation.

Discussion

As per our knowledge, this is the first report describing the morphology of urinary exosomes from buffaloes at estrus phase. According to Neerukonda *et al*. (2020), PEG may have contributed to the exosome co-precipitation seen in Fig. 1C. However, the present study has the limitation of describing the presence of exosome markers by immunostaining, nanoparticle tracking analysis (NTA) (Street *et al*., 2017) or western blot for exosome associated abundant proteins (Koh *et al*., 2017) (membrane transport and fusion (GTPases, annexins and flotillins); multivesicular body biogenesis (ALIX, TSG101 and clathrin); tetraspanins (CD9, CD63, CD81 and CD82) and heat shock proteins (HSC70 and HSP90). Nevertheless, the present protocol of standardization of PEG precipitation for the isolation of urinary exosomes form buffalo species provides a rapid, scalable, and efficient substitute for the ultracentrifugation method of isolating exosomes from buffalo urine having advantages such as accessibility to benchtop centrifuge, cost-effectiveness, ability to handle large sample volumes and higher recovery rates.

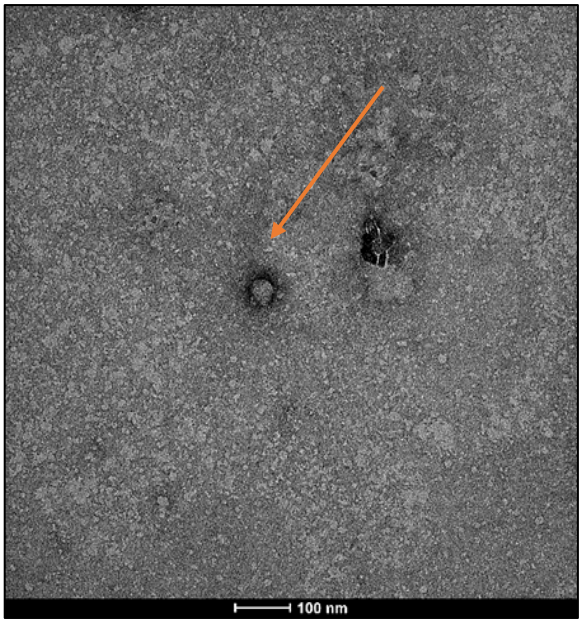

Fig. 1A

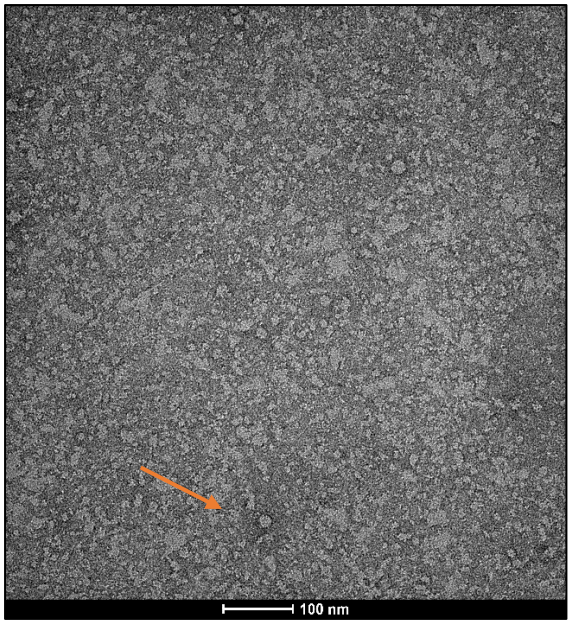

Fig. 1B

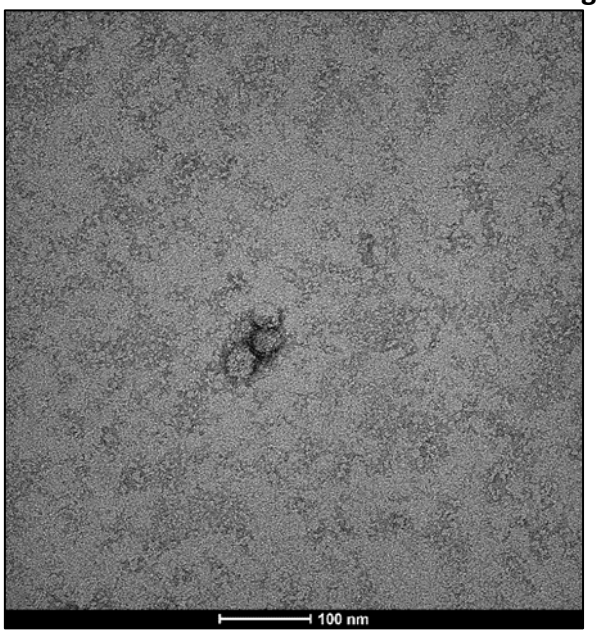

Fig. 1C

Fig. 1: 1A, 1B and 1C show the exosomes found in buffalo estrus urine samples precipitated by PEG based method (Size of vesicles indicated in the bar down)

From the urinary exosome protein bands resolved on SDS-PAGE, it is evident that PEG based method as described in this manuscript yielded more protein when compared to the ultracentrifugation (as shown by the protein bands' intensity in Fig. 2). The urinary exosomes isolated by ultracentrifugation were according to the procedure described by He *et al*. (2019) with slight modifications. The yielded protein bands in urinary exosomes isolated by PEG based method can be attributed to the co-precipitation. The protein pellet of urinary exosomes obtained in the present study can be resuspended in rehydration buffer (7M Urea, 2M Thiourea and 2% CHAPS) for resolving the protein bands (which can be of low abundance and not resolved on SDS-PAGE) precisely from various biological groups (e.g. for differentially expressed proteins in diestrus, regular estrus and silent estrus buffaloes) by proteomic tools like two-dimensional gel electrophoresis (2D- PAGE) (Zubiri *et al*., 2013) or mass spectrometry (MS)-based proteomic analyses (Erozenci *et al*., 2019).

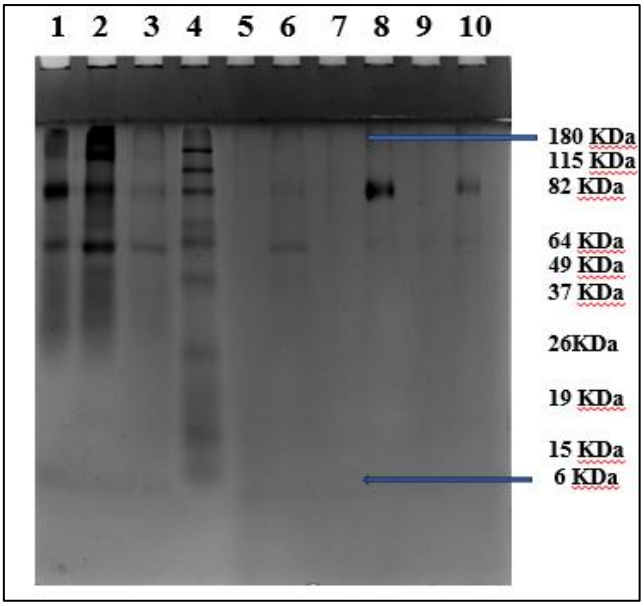

Fig 2: Lane 1, 2 and 3 shows the buffalo urinary exosome protein (isolated by PEG based method followed by Trizol method) bands resolved on SDS-PAGE from silent estrus, diestrus and regular estrus samples respectively; Lane 4 shows the protein ladder (BenchMark Prestained protein ladder); Lanes 6, 8 and 10 shows the buffalo urinary exosome protein (isolated by ultracentrifugation followed by trizol method) from silent estrus, diestrus and regular estrus samples respectively.

Downstream, after the characterization of exosome markers, urinary exosome proteome and transcriptome studies can be initiated which can have great implications for biomarker discovery (Lv *et al*., 2018). Further, the compatibility of total RNA extracted from urinary exosomes by PEG based method described in the present study for miRNA expression studies by Q RT-PCR can be explored in future.

Conclusion

Urinary exosomes are a promising non-invasive and easily accessible source of biological material to investigate biomarkers. We conclude that the PEG based precipitation method described in this manuscript is a simple and efficient way to isolate urinary exosomes and can be an alternative to the ultracentrifugation method which is labour-intensive and requires expensive equipment. Though there is a limitation in the present study that the urinary exosomes isolated by the PEG based precipitation method has not been validated for proteomic and transcriptomic studies, the yield of protein seems to be higher when compared to the ultracentrifugation method which can be an added advantage.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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